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Analysis of immunoglobulin variable region genes of a human IgM anti-myeloperoxidase antibody derived from a patient with vasculitis

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SUMMARY

Circulating antibodies to myeloperoxidase (MPO) are associated primarily with pauci-immune glomerulonephritis and systemic vasculitis. Anti-MPO antibodies belong to a group of autoantibodies, anti-neutrophil cytoplasmic antibodies, that may play a pathogenic role in vasculitis. We have generated a human monoclonal anti-MPO antibody (E3-MPO) using peripheral blood lymphocytes from a patient with microscopic polyarteritis. Variable region gene analysis of E3-MPO showed that the V_H region had 90% homology with the germ line gene V_H4-21. E3-MPO was also shown to carry the 9G4 idiotope, which so far has been associated only with human antibodies that utilize the V_H4-21 gene. The 9G4 idiotope was also expressed on anti-MPO antibodies in sera from the donor patient and from 4/7 additional patients with active, untreated vasculitis. The nucleotide sequences of both the variable heavy and light chains of E3-MPO showed evidence of an antigen-driven response.

INTRODUCTION

Circulating anti-myeloperoxidase (MPO) antibodies are associated largely with pauci-immune glomerulonephritis and microscopic polyarteritis, but are present occasionally in sera from patients with other autoimmune diseases.[1] Anti-MPO antibodies only recognize the native form of the enzyme.[2] Human poly- and monoclonal antibodies and murine monoclonal antibodies to MPO do not appear to bind near the active site, as they do not affect the activity of the isolated enzyme.[3] Anti-MPO antibodies may, however, interact with MPO when it is present on the membrane of pre-primed neutrophils, resulting in degranulation and the production of cell-damaging free radicals *in vitro*.[4] Antibodies to granule enzymes such as MPO may therefore play a proinflammatory role in vasculitis through a direct effect on neutrophils at susceptible sites.

Both IgM and IgG anti-MPO antibodies have been detected in sera from patients with active disease; however, high circulating levels of anti-MPO antibodies are not invariably associated with active disease.[5] The pathogenic potential of anti-MPO antibodies may therefore be closely related to particular characteristics of different populations of anti-MPO antibodies during the evolution of the disease. The idiotypes expressed on anti-MPO antibodies have not yet been defined by specific reagents, although anti-idiotypic activity has been detected in sera from patients in remission.[6]

Analyses of the variable region genes that encode other autoantibodies have shown that some, generally of the IgM class, have complete identity with their germ line genes of origin, whereas others, particularly of the IgG class, are somatically hypermutated. Determining the amino acids that constitute the complementarity determining regions (CDR) of immunoglobulin variable regions has also offered clues to the way in which they bind to autoantigens.[7] In some cases, the mutational patterns show evidence of an antigen-driven response. In this study we have characterized the immunoglobulin variable region genes encoding a previously described human IgM monoclonal antibody to MPO derived from a patient with vasculitis.[8]

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MATERIALS AND METHODS

Patients

Eight untreated patients with a clinical diagnosis of vasculitis according to the 1990 American Rheumatology Association criteria,[9] and with renal biopsy findings of necrotizing glomerulonephritis and absent immune deposits, were studied. Their sera all showed a perinuclear staining pattern on ethanol-fixed human neutrophils and were positive for anti-MPO antibodies by enzyme-linked immunosorbent assay (ELISA). The clinical details of the patients studied and binding of their sera to MPO are summarized in Table 1.

Table 1. Clinical characteristics of patients with vasculitis

Patient no.	Age (years)	Gender	Organ involvement			Anti-MPO*	
			Kidney	Lung	Skin	IgM	IgG
1†	62	M	+	+	–	0·930	0·670
2†	42	M	+	+	–	0·018	0·860
3	46	M	+	+	–	0·200	0·829
4†	62	M	+	+	–	0·779	1·139
5†	78	M	+	–	–	1·810	0·130
6	73	F	+	+	+	0·028	0·861
7	73	F	+	+	–	0·139	1·347
8†	52	F	+	+	–	0·205	1·300

E3-MPO was derived from patient 1.

*OD values (405 nm) in ELISA to detect anti-MPO antibodies. Values given are those obtained following incubation with substrate at room temperature for 30 min. Background values given by samples on the uncoated side of an ELISA plate have been subtracted.

†Indicates patients whose circulating anti-MPO antibodies expressed the 9G4 idiotope.

Production of human hybridomas

The human hybridomas were produced from the fusion of peripheral blood lymphocytes from a patient with microscopic polyarteritis (patient 1; Table 1) and the CB-F7 heteromyeloma cell line, as described previously.[8] The IgM monoclonal anti-MPO antibody, E3-MPO, which bound to the native form of myeloperoxidase, was selected for variable region gene analysis.

Detection of anti-MPO antibodies by ELISA

A previously described direct binding ELISA using purified human leucocyte MPO was used to detect IgM and IgG anti-MPO antibodies.[10]

Sequence analysis

Total RNA was extracted from the hybridoma cell line E3-MPO using the guanidium isothiocyanate/caesium chloride method.[11] One microgram of total RNA was used to make cDNA utilizing Moloney murine leukaemia virus reverse transcriptase (Gibco BRL, Paisley, UK) and random hexamer primers.[12] The immunoglobulin variable regions were then amplified by polymerase chain reaction (PCR) using a variety of family-specific primers to amplify either the V_H gene families 1–6 and Vκ genes. PCR products were only obtained with primers specific for the V_H4 or Vκ1 families. PCR conditions were as follows. The V_H region was amplified using primers specific for the V_H4 heavy chain leader (5′-ATGAAACACCTGTGGTTCT) at the 5′ end and the Cμ primer (5′-CGAGGGGGAAAAGGGTTGG) at the 3′ end. The primers used for amplification of the Vκ region were (5′GACATCCAGATGACCCAGTCTCC 3′) at the 5′ end and the κ constant region-specific primer (5′CGAGAATTCTAGATCATCAGATGGCGGA 3′) at the 3′ end. PCR amplification for V_H and Vκ regions consisted of an initial denaturation step of 5 min at 94°, followed by 30 cycles of 1 min at 94°, 1 min at 56° and 1 min at 72°, with a final extension time of 5 min at 72°. The amplified products were electrophoresed through a 1·5% agarose gel and purified using Geneclean II (Bio 101 Inc., La Jolla, CA). The purified DNA from the V_H and Vκ regions was cloned into a TA cloning vector (Novagen, Madison, WI) and transfected into *Escherichia coli* strain JM109 (Promega, Maddison, WI). Single-stranded (SS)DNA was obtained from positive clones using VCS-M13 helper phage (Strategene, La Jolla, CA) and sequenced via the dideoxy chain termination method using the Sequenase kit (USB, Cambridge Bioscience, Cambridge, UK). Sequencing was performed on ten clones derived from two independent PCR products to check for amplification error. The nearest germ line counterparts expressed in E3-MPO were determined using the V BASE sequence directory (I. Tomlinson, MRC Centre for Protein Engineering, Cambridge, UK). Due to the death of the patient, it was not possible to perform a direct germ line gene analysis.

Expression of 9G4 idiotope on anti-MPO antibodies

Human leucocyte MPO was coated onto ELISA plates at a concentration of 1 μg/ml in sodium bicarbonate buffer (pH 9.6). Following incubation overnight at 4°, and blocking with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), E3-MPO-containing supernatants at a concentration of 0·5 μg/ml or patient sera diluted 1/100 with PBS/0·05% Tween-20, pH 7·3, were added to duplicate wells. Sera from five normal controls were also included. This, and all subsequent incubations, were at 37° for 1 hr. Washing between each step was with PBS/0·05% Tween-20. The rat monoclonal antibody 9G4 was then added at a concentration of 3 μg/ml. The plates were developed using an anti-rat biotin reagent (Sigma Chemical Co., Poole UK), followed by alkaline phosphatase-conjugated strepavidin with p-nitrophenol phosphate used as substrate. Optical density (OD) at 405 nm was measured following incubation for 30 min at room temperature. A positive reaction for 9G4 expression by antibodies binding immobilized MPO was defined arbitrarily as those samples giving an OD > 0·2 (following subtraction of background binding of sera to uncoated wells).

RESULTS

Variable region analysis of the heavy chain

The nucleotide sequence of the V_H region of the IgM anti-MPO

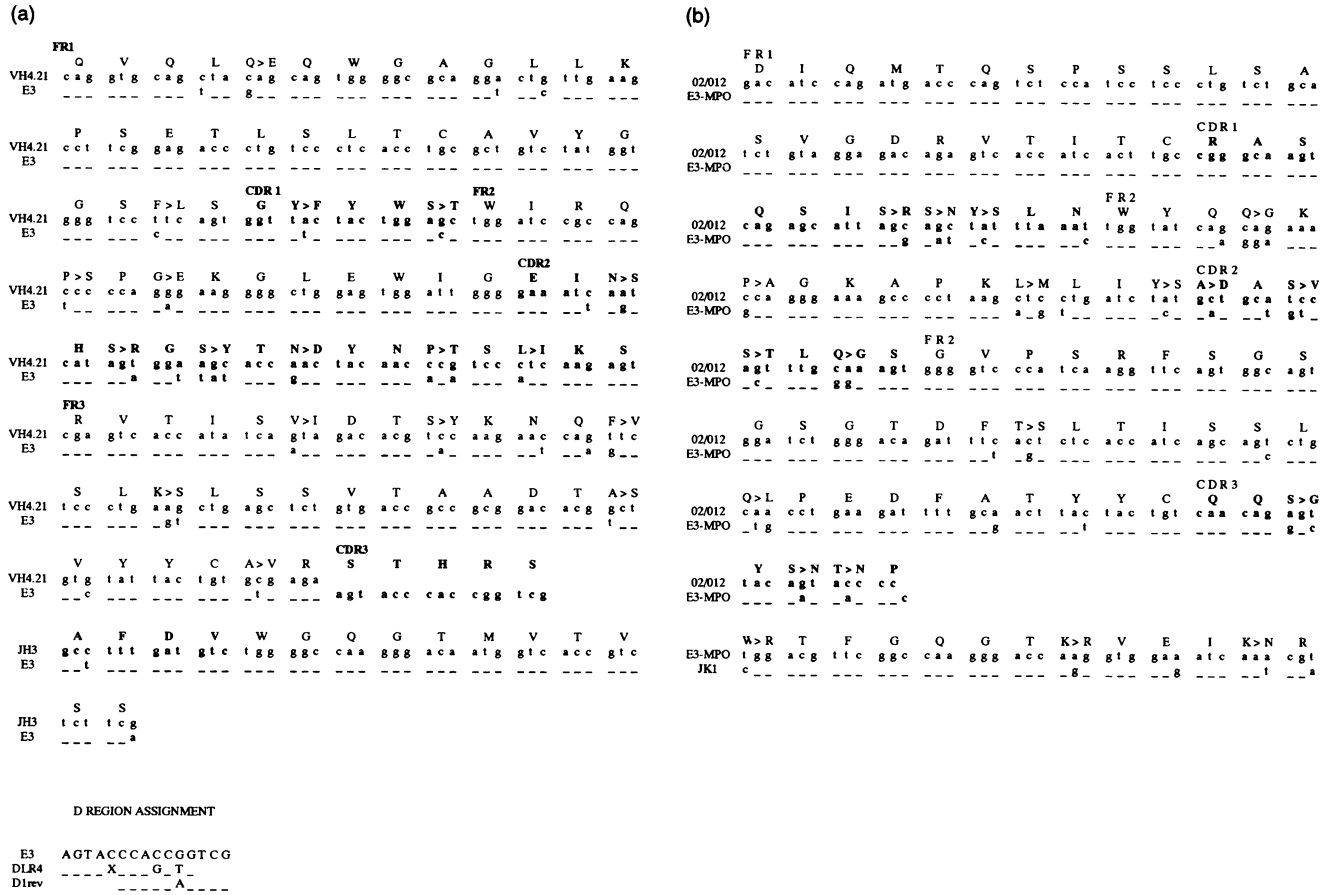

Figure 1. V_H and V_L nucleotide sequence of E3-MPO. (a) The V_H gene is shown aligned to the V_H4-21 germ line gene with the D-region assignment shown below. (b) The V_L gene of E3-MPO is aligned to the germ line gene 02/012. Identities of nucleotides are shown by dashes and differences shown at the respective positions. Amino acid designations for the codons are shown at the top of the nucleotide sequence and replacement mutations are also indicated.

antibody E3-MPO showed that it had 90% homology with the germ line gene V_H4-21 at the nucleotide level (Fig. 1a). Although further tissue could not be obtained from the donor to determine the exact germ line gene of origin, the V_H4-21 gene is not thought to be polymorphic.[13] The D region was aligned to two D gene segments, DLR4 and D1, and an unmutated J_H3 gene was utilized.

In the framework regions, a total of nine replacement mutations was present with only two resulting in a change to a negatively charged residue. The CDR regions were highly mutated, with 11/23 codons affected, nine of these changes resulting in replacement mutations, two of which involved the gain of a charged residue. The CDR3 region was notably short, containing only five amino acids, one of which was the negatively charged aspartic acid, and two were positively charged, namely histidine and arginine.

The nucleotide sequence of the E3-MPO light chain variable region had 90% homology with the Vκ1 germ line gene 02/012 (Fig. 1b). A Jκ1 gene segment was utilized with four replacement mutations. Framework regions two and three had a total of six replacement mutations, none of which resulted in any change in the number of charged residues. Within the CDR, mutation away from the germ line was most striking in 13 of the codons affected, 10 of which resulted in amino acid replacements and the gain of three charged residues.

The ratio of replacement to silent mutations (R/S) in the CDR domains of the V_H region of E3-MPO was 2·25 : 1, and in the light chain 3 : 1. These calculations were made assuming the germ line genes of origin were V_H4-21 for the heavy chain and 02/012 for the light chain. Analysis of the distribution of replacement mutations within the CDR of the heavy and light chains of E3-MPO using the binomial distribution method of Chang & Cassali (1994) gave values of $P = 0{\cdot}041$ and $P = 0{\cdot}009$, respectively. These probability values suggest that there is a high degree of mutation away from the germ line and would therefore indicate that the antibody arose by an antigen-driven mechanism.

Expression of 9G4 idiotope

In Fig. 2, the binding of the rat monoclonal antibody 9G4 to E3-MPO and to anti-MPO antibodies present in sera from eight patients with vasculitis is shown. E3-MPO and anti-MPO antibodies in 5/8 sera from vasculitis patients were found to express the 9G4 idiotope (OD > 0·2). There was a significant correlation between IgM anti-MPO antibody levels, but not

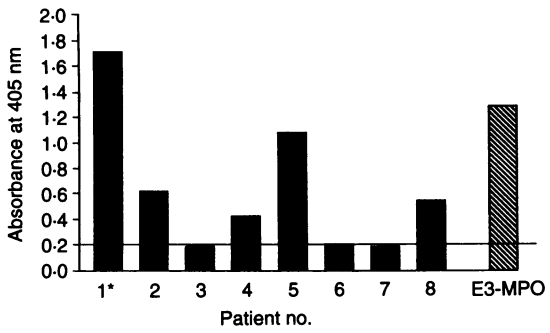

Figure 2. Expression of the 9G4 idiotope on E3-MPO and on circulating anti-MPO antibodies in sera from eight patients with vasculitis. This figure shows the reactivity of the 9G4 anti-idiotypic antibody with anti-MPO antibodies from the sera of eight patients with vasculitis (Table 1) and on E3-MPO (immobilized by binding to MPO coated onto microtitre plates), using an ELISA. Anti-MPO antibodies in 5/8 vasculitis sera and E3-MPO significantly bound the 9G4 reagent.

IgG anti-MPO antibody levels, and the expression of 9G4 ($P < 0{\cdot}05$; data not shown).

Comparison of the amino acid sequence of E3-MPO with other V_H4-21-derived autoantibodies

E3-MPO did not agglutinate red cells at a concentration of 1 μg/ml (data not shown), nor did it react with ssDNA.[8] The amino acid sequence of E3-MPO was compared with that of other V_H4-21-encoded autoantibodies (Fig. 3). E3-MPO was found to show a more pronounced clustering of mutations in the CDR2 domain. The 9G4 reagent is thought to bind to the AVY motif at the end of FR1 (shown in italics in Fig. 3). This sequence was conserved in E3-MPO.

DISCUSSION

We have described the nucleotide sequence of the first human monoclonal antibody to myeloperoxidase. The heavy chain showed 90% homology to the germ line gene V_H4-21. V_H4-21 is likely to be the germ line gene of origin, as E3-MPO expressed the 9G4 idiotope. To date, 9G4 idiotope has only been found on antibodies utilizing V_H4-21.[15] The number of replacement mutations, particularly in the CDR of the V_H and V_L domains of E3-MPO, suggested that an antigen-driven response may play a role in the generation of this autoantibody.

The expression of the 9G4 idiotope both on E3-MPO and anti-MPO antibodies in sera from patients with vasculitis suggests that at least a proportion of these autoantibodies (predominantly of the IgM class) was derived from the V_H4-21 gene segment. This could indicate a common origin for the development of anti-MPO antibodies in different patients. Interestingly, some patients with vasculitis had only IgM anti-MPO antibodies detectable in their sera (e.g. patient 5; Table 1), and high circulating IgM anti-neutrophil cytoplasmic antibodies (ANCA) have been associated with pulmonary haemorrhage.[16] The 9G4 idiotope is also expressed on human antibodies with cold agglutinin activity which have I/i specificity,[17] and V_H4-21 gene usage appears mandatory for these antibodies. The 9G4 idiotope has also been found on human monoclonal anti-DNA antibodies and rheumatoid factors, expressed on immunoglobulin deposits in kidney biopsies from lupus patients, and found to be elevated in the sera of systemic lupus erythematosus (SLE) patients.[18] Although the question as to whether there is a bias towards the usage of certain germ line gene families in autoimmune disease is controversial,[19,20] our findings provide another example of human autoantibodies utilizing V_H4-21 (in this case with specificity for MPO), suggesting a clinically important role for this V_H gene product.

Sequence analysis of V_H4-21 has shown that 9G4 recognizes an idiotope in the FR1 region product of this gene.[13] Comparison of the nucleotide sequence of E3-MPO with a bank of V_H4-21-encoded cold agglutinins revealed a highly conserved first framework region as a common feature, and may account for its ability to react with 9G4 (Fig. 1a). E3-MPO has only one replacement mutation within FR1 but it is near the beginning of this region. Other areas of the sequence, however, are highly mutated (particularly the CDR2 region) and, interestingly, E3-MPO has a shorter CDR3 than the cold agglutinins. These differences may explain the fact that E3-MPO did not agglutinate red cells.[21]

The conformational epitope on MPO to which E3-MPO

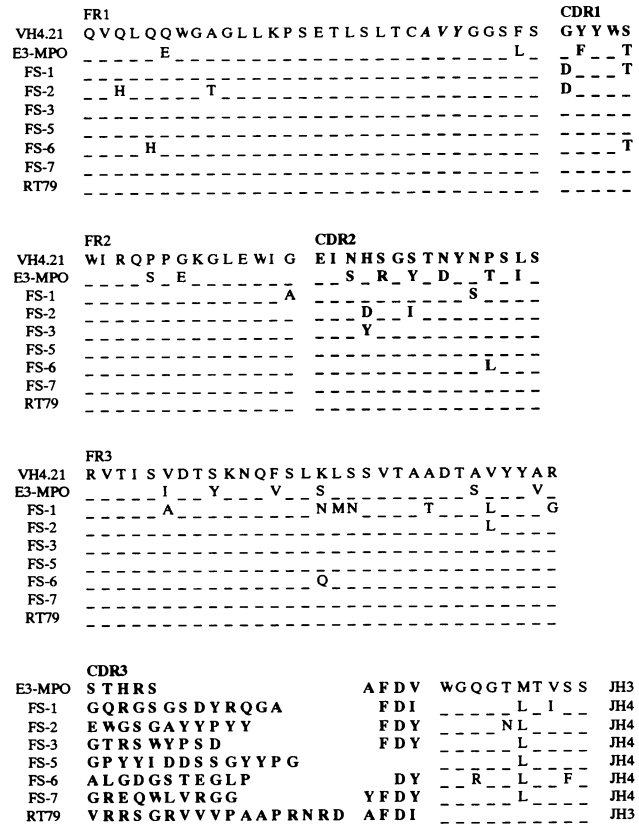

Figure 3. Deduced amino acid sequence of the V_H of E3-MPO compared with that of the germ line gene V_H4-21 and seven other human monoclonal autoantibodies. The amino acid sequence of E3-MPO is compared with that of seven human monoclonal autoantibodies derived from V_H4-21 (all six FS designated antibodies had cold agglutinin activity and RT79 reacted with ssDNA). The AVY amino acid motif in the first framework region, recognized by the anti-idiotope reagent 9G4, is shown in italics. Identity with germ line sequence is indicated with dashes.

binds has not been defined but it is possible that local charge may be important. There is evidence to suggest a role for positively charged residues in conferring autoantibody binding to negatively charged DNA.[22] When considering the binding of antibodies to positively charged MPO (pI = 11·0), negatively charged amino acid residues in the V regions, for example, could aid binding to MPO or increase binding affinity. Examination of the derived amino acid sequence of E3-MPO, however, did not reveal any unique features of the linear amino acid sequence that would indicate that changes to negatively charged residues are important in the binding of E3-MPO to its antigen. This is perhaps not surprising as MPO is a large (~135 000 MW) globular protein that may have localized concentrations of charge despite an overall positive charge, whereas DNA consists of relatively uniform, negatively charged repeating units. Only analysis of the tertiary structure will reveal which amino acids are exposed at the antigen-binding site and may be involved in binding to MPO.

The origin of antibodies to neutrophil granule proteins is not known, although they are unlikely to arise as merely the consequence of neutrophil degranulation and sequestration. This study describes the first sequenced human IgM monoclonal antibody to MPO. E3-MPO showed evidence for antigen selection, being mutated away from its deduced germ line gene of origin, V_H4-21. In addition, the expression of the 9G4 idiotope on anti-MPO antibodies in sera from other newly presenting patients with vasculitis suggests that usage of V_H4-21 is common in circulating anti-MPO antibodies.

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